STUDY ON THE MET-tRNA-BINDING ACTIVITY IN KREBS-2 CELLS INFECTED WITH ENCEPHALOMYOCARDITIS VIRUS

A. M. DIZHOOR and T. Yu. UGAROVA

Department of Virology and *A. N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR*

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1. Introduction

Infection of mammalian cells with picornaviruses causes preferential inhibition of host-cell protein synthesis at the early stages of infection and depression of both cell- and virus-specific protein synthesis at the late stages [1]. The preferential inhibition of cell protein synthesis in the first half of the infection cycle in Krebs-2 cells infected with encephalomyocarditis virus (EMCV) is masked by intensive EMCV-specific protein synthesis, the total level of protein synthesis being unaffected [2]. We have reported that during this period the level of the Met-tRNA$_f$ · 40 S ribosomal subunit preinitiation complex formation in the infected cells does not differ from that in controls. The inhibition of the preinitiation complex formation develops at the later stages of infection in parallel with decrease in the overall protein synthesizing activity of the infected cells [2].

It was of interest therefore to investigate the activity of the initiation factor eIF-2 in order to find out whether or not its inactivation contributes to the impairment of the host-cell protein-synthesizing machinery in EMCV-infected Krebs-2 cells. The modification of this factor resulting in a decrease of the 40 S preinitiation complex formation is generally believed to be a widespread cause of inhibition of protein synthesis in eukaryotic cells, e.g., in reticulocytes in the absence of hemin and under some other conditions [3–6].

These experiments show that the inhibition of both the protein synthesis and the preinitiation complex formation in EMCV-infected Krebs-2 cells is not due to inactivation of eIF-2, since the formation of both the eIF-2 · GTP · Met-tRNA$_f$ ternary complex and the Met-tRNA$_f$ · 40 S preinitiation complexes in cell-free extracts isolated from infected cells at the late stages of infection proceeds with a normal efficiency.

2. Methods

2.1. *Infection of the cells*

Ascites carcinoma Krebs-2 cells maintained in mice were washed 3 times with a cold Earle's solution and infected with EMCV at a multiplicity of 20 p.f.u./cell. For synchronization of infection the cells were incubated at 4°C overnight. Then the infected cells were incubated at 2×10^7 cells/ml in Eagle's medium at 37°C.

2.2. *Isolation of the postmitochondrial fraction*

The cells were washed with a cold buffer containing 30 mM Tris–HCl (pH 7.6) and 145 mM NaCl, suspended in 2 vol. 10 mM Tris–HCl (pH 7.6), 10 mM KCl, 1.5 mM $MgCl_2$ for 30 min at 0°C, and disrupted in a Dounce homogenizer; the homogenate was immediately supplemented with 1/9 vol. buffer containing 200 mM Tris–HCl (pH 7.6), 1 M KCl, 37 mM $MgCl_2$. Fraction S_{10} was obtained after centrifugation in an MSE 8 × 50 rotor for 15 min at 10 000 rev./min at 4°C.

2.3. *Isolation of crude initiation factors*

Ribosomes were isolated and washed with 0.5 M KCl as in [7]. All the steps of the procedure of preparation of the factors were carried out at <4°C. The factors were precipitated with solid $(NH_4)_2SO_4$ added to 70% saturation at 0°C with constant stirring. 30 min after $(NH_4)_2SO_4$ addition, the precipitate was collected by centrifugation, dissolved in 30 mM Tris–

HCl (pH 7.6), 100 mM KCl, 10 mM β-mercaptoethanol with 10% (v/v) glycerol, and the solution was dialyzed overnight against the same buffer. After removal of the precipitate, the solution that contained initiation factors was frozen in small portions in liquid nitrogen and stored at –60°C.

2.4. *Preparation of [^{3}H]Met-tRNA$_f$*

tRNA$_f$ was isolated from the preparation of total tRNA by chromatography on BD-cellulose and charged with [^{3}H]methionine as in [8]. The specific radioactivity of the partially purified [^{3}H]Met-tRNA$_f$ was 13 500 cpm/μg.

2.5. *Millipore binding assay of eIF-2 · GTP · Met-tRNA$_f$ ternary complex formation*

The formation of the ternary complex in vitro was assayed as in [9]. The reaction mixture contained (per 0.05 ml) 30 mM Tris–HCl (pH 7.6), 100 mM KCl, 7 mM β-mercaptoethanol, 0.2 mM GTP, [^{3}H]-Met-tRNA$_f$ (67 000 cpm) and crude initiation factors. After 7 min incubation at 37°C, the samples were diluted with an ice-cold buffer containing 20 mM Tris–HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$ and passed through Millipore filters, type HA. The filters were washed 3 times with the same buffer, dried and the radioactivity of the adsorbed material was counted.

2.6. *Formation of the 40 S preinitiation complex in vitro*

A cell-free system contained (per 0.1 ml) 60 μl S_{10} fraction, 30 mM Tris–HCl (pH 7.6), 65 mM KCl, 3.3 mM $MgCl_2$, 1.5 mM ATP, 10 mM creatine phosphate, 4 μg creatine phosphokinase, 0.2 mM GTP, 2 μCi [^{3}H]methionine (Amersham) and 100 μg cycloheximide/ml. After 2 min incubation at 30°C, the reaction mixture was diluted 10-fold with an ice-cold buffer containing 15 mM Tris–HCl (pH 7.6), 50 mM KCl and 2 mM $MgCl_2$ and layered on a 15–30% sucrose concentration gradient. After 16 h centrifugation in a Spinco SW 27 rotor at 24 000 rev./min at 3°C, the radioactive material was precipitated from the gradient fractions by cetyltrimethylammonium bromide as in [10].

3. Results and discussion

To estimate the level of the functional activity of eIF-2 at the late stages of infection (4.5–5.5 h post-infection), we have investigated both eIF-2 · GTP · Met-tRNA$_f$ ternary complex formation and Met-tRNA$_f$ · 40 S preinitiation complex formation in preparations obtained from the infected as well as non-infected cells.

Table 1
[^{3}H]Met-tRNA$_f$-binding activity in a crude initiation factor (eIF$_s$) preparation from Krebs-2 cells

Reaction mixture	[^{3}H]Met-tRNA$_f$ binding (cpm)
Complete	2100
– eIF$_s$	200
Complete + 3 mM GDP	200
Complete + 3 mM $MgCl_2$	650

A complete system contained 30 μg eIF$_s$, 23 000 cpm [^{3}H]-Met-tRNA$_f$, 3 mM GTP and other components as in section 2.5. The samples were incubated and treated as in section 2.5

Preliminary experiments showed that some characteristics of the Met-tRNA$_f$-binding activity in the crude initiation factors preparation from Krebs-2 cells were similar to those of the eIF-2 (see table 1). The binding of [^{3}H]Met-tRNA$_f$ to nitrocellulose filters promoted by crude initiation factors is reduced by added Mg^{2+} [15] and is completely suppressed by GDP. The millipore binding assay of the ternary complex formation indicates that even at 5.5 h postinfection the Met-tRNA$_f$-binding activity of eIF-2 is not reduced (fig.1).

In vitro formation of the 40 S preinitiation complex was investigated using an S_{10} fraction obtained

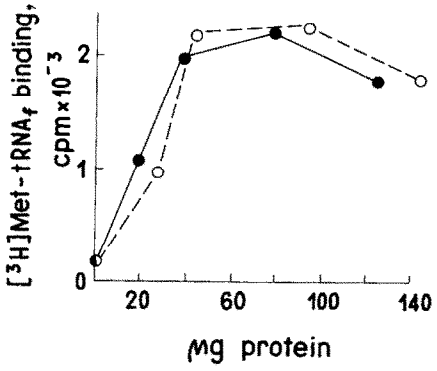

Fig.1. Millipore binding assay of ternary complex formation. Crude initiation factor preparations isolated from mock-infected (•) and EMCV-infected (○) cells 5.5 h postinfection were incubated with [^{3}H]Met-tRNA$_f$ in the presence of GTP as in section 2.5.

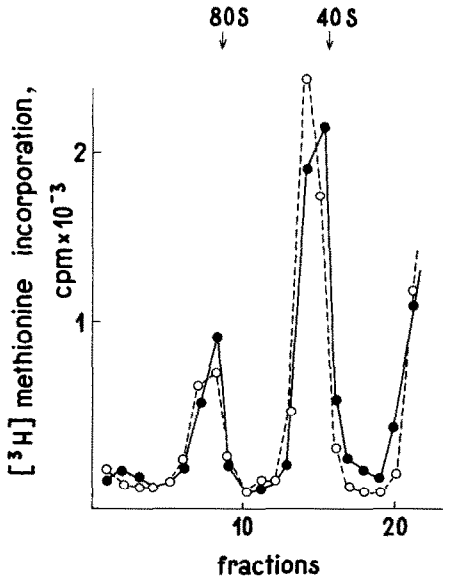

Fig.2. In vitro formation of the 40 S preinitiation complex. S_{10} fractions isolated from mock-infected (•) and (○) EMCV-infected cells were incubated with [^{3}H]methionine for 2 min in the presence of cycloheximide as in section 2.6. The samples were analyzed by centrifugation in a sucrose concentration gradient.

from the cells after 4.5 h infection. Postmitochondrial fractions prepared from both control and EMCV-infected cells demonstrate the same ability to form the 40 S complex (fig.2).

There is good agreement between the results presented here and other data. In [11] the ability of native 40 S ribosomal subunits to bind Met-tRNA$_f$ did not change in Mengo virus-infected L cells. Evidence for the eIF-2 functional stability in poliovirus-infected HeLa cells has also been reported [12].

According to [13], the 40 S preinitiation complex formation in EMCV-infected HeLa cells declines in parallel with inhibition of total protein synthesis at the late stages of infection. In this case, the in vitro formation of the 40 S complex in cell-free extracts prepared from the infected cells was depressed as well, which is at variance with these results. The reason for this phenomenon is not immediately apparent, but the inactivation of eIF-2 might well occur during the preparation of cell-free extracts in [13], as postulated by the authors themselves.

These results suggest the absence of any irreversible changes in the eIF-2 activity in Krebs-2 cells infected with EMCV. Therefore, neither the decrease in the level of the in vivo 40 S preinitiation complex formation nor the inhibition of overall protein synthesis observed at the late stages of infection in this case is associated with the modification of eIF-2.

It is known that picornaviral infection leads to changes in the intracellular ion concentrations [14]. Therefore there is a possibility that the inhibition of 40 S preinitiation complex formation observed in EMCV-infected Krebs-2 cells in vivo is associated with perturbance in the ion balance, rather than with irreversible changes in the components of the translation system.

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